EFFECT OF TEMPERATURE ON CATALYTIC HYDROGEN CURRENTS OF NATIVE AND MODIFIED BOVINE SERUM ALBUMIN

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Dedicated to the late Professors J. Heyrovský and R. Brdička.

The effect of temperature has been studied on three different catalytic hydrogen currents observed voltammetrically and on two of them polarographically with serum albumin and modified products of albumin adsorbed on mercury. The so-called "active cobalt catalytic current", i_c and "presodium current", i_{ps} increase with increasing temperature. The temperature effect on the so-called Brdička currents. i_1 and i_2 was found to be quite different from that on i_c or i_{ps}. In ammoniacal buffer (pH = 9·3) in the presence of cobalt(III) or (II) i_1 has been found virtually the same at temperatures between 4° and 40°C, whereas i_2 greatly decreases with increasing temperature. Evidence has been presented that in the presence of Co(III) or Co(II) and at 4°C all disulfide groups in the proteins used are reduced to sulfhydryl at potentials at which i_1 and i_2 are observed. It has been concluded that the ligands of the protein which complex with Co(III) or Co(II) or Co(0) are different at potentials at which i_1 is observed than at which i_2 is observed. In order to account for the abnormal temperature effect on i_2 it has been proposed that the area of the section of protein which is the seat of i_2 is being detached from the surface with increasing temperature and that this process is reversible.

Three kinds of catalytic hydrogen currents of native or modified albumin can be observed in alkaline buffer solutions at the hanging mercury drop electrode (HMDE). Since the discovery by Brdička[1] of catalytic hydrogen currents at the dropping mercury electrode (DME) of sulfhydryl or/and disulfide containing compounds, including proteins, in ammoniacal buffers in the presence of cobalt(III) hexammine chloride [Co(III)] or cobalt(II) chloride [Co(II)] more than one thousand papers have been published dealing with these currents, referred to in the literature as Brdička currents. Quite generally, with sulfhydryl and/or disulfide containing proteins two more or less well defined Brdička currents, referred to by i_1 and i_2 in this paper, are observed. Recently[2] characteristics of these currents observed with bovine serum albumin (BSA) at the hanging mercury drop electrode (HMDE) have been described. In spite of the abundant literature no quantitative interpretation of the effect of various factors which determine Brdička currents observed at the DME or HMDE has been given. A qualitative interpretation of these effects, involving a chain mechanism has been proposed[2].

Also many years ago, Herles and Vančura[3] discovered that proteins (containing —SH and/or —S—S—) yield large catalytic hydrogen currents at the DME in ammoniacal buffer solutions both in the absence as well as in the presence of Co(III) or (II). These currents have been called "presodium currents" by their discoverers, as they are observed in sodium containing buffers at slightly less negative potentials than those of sodium waves. We will refer to these currents using the symbol i_{ps}. These currents, in general, are well defined at the HMDE[2].

More recently a different type of catalytic hydrogen current, referred to by symbol i_c, has been discovered in this laboratory[4]. It is observed at the HMDE, (but not at the DME), when "active cobalt" is deposited on the HMDE which contains a trace of adsorbed sulfhydryl or disulfide containing compound. Anzenbacher and Kalous[5] discovered that when a HMDE, in an ammoniacal buffer containing low molecular weight thiol or disulfide and Co(II), was kept breifly at a potential of about —1·0 V (*vs* S.C.E.) and then recycled anodically the "active cobalt" yielded two anodic waves with peaks at —0·25 V and —0·05 V. In a buffer which is 0·1M in ammonia and which contains Co(III) or (II) we have observed at the HMDE containing traces of adsorbed albumin after deposition of "active cobalt" a catalytic hydrogen current, i_c, with a peak at about —1·45 V (*vs* S.C.E.)[4]. The anodic scanning (after deposition of active cobalt) must be followed within a few seconds by the cathodic scanning. When a HMDE was placed for 2 h at 21°C in the stirred buffer which was only $5 \cdot 10^{-12}$M in BSA (ref.[4]) and $5 \cdot 10^{-4}$M Co(III) or Co(II) the value of i_c was 82—20 = 62 μA, the figure of 20 μA being the current found after 2 h in the blank without BSA.

The objective of the present paper is the determination under various conditions of the effect of temperature on Brdička currents (i_1 and i_2), on i_{ps} and on i_c of bovine serum albumin (BSA) and of modified products of albumin. Brdička currents and i_{ps} were determined polarographically at the DME and voltammetrically at the HMDE. For obvious reasons i_c can be observed only at the HMDE and not at the DME under ordinary polarographic conditions.

EXPERIMENTAL

The chemicals and products of BSA and characteristics of the hanging mercury drop electrode have been described previously[2,4], in which references to previous publications have been made).

The native albumin used contained 17 —S—S— groups and less than one —SH and is denoted by P[—SH, =[S—S]$_{17}$]. Modified products of BSA are denoted by P(SH)$_{35}$, P[—(SCH$_2$)COONa)$_{35-x}$, —(SCH$_2$)$_x$], P[—(SH$_2$COONa)$_{35-2y}$, =(S—S)$_y$] and P[—(SH)$_x$, =(S—S)$_{17-x/2}$]. Their preparation has been described previously[4]. The scan rate in voltammetry was 500 mV/s and the drop surface of the HMDE was $2{\cdot}23 \cdot 10^{-2}$ cm^2.

RESULTS

Brdička Currents

Voltammetry at the HMDE

Procedure. Method *1*: The HMDE was kept for a given time and at a given temperature in the protein containing buffer which was 0·1M in ammonia and 0·1M in ammonium chloride (pH = 9·3) and unless stated otherwise $5 \cdot 10^{-4}$M in Co(III) or (II). The voltammogram was run at the same temperature. Method *2*: The HMDE was kept for a given time and at a given temperature in the protein containing buffer (in the absence of cobalt). The HMDE was then placed for 30 s in the protein-free buffer and then in the buffer with Co(III) or (II) at a given temperature.

The results are in Table I and the characteristics of the voltammograms at different temperature are illustrated in Fig. 1. The monolayer on the HMDE was formed at concentrations of BSA varying between $2\cdot5 \cdot 10^{-7}$ and $2\cdot5 \cdot 10^{-8}$M. As expected, the time required to form a monolayer was found to decrease with increasing concentration and increasing temperature. In order to get information whether the decrease of i_2 with temperature can be attributed to an irreversible "heat" denaturation, all experiments were repeated by carrying out the adsorption at 4°, 21° and 35°C and then running the voltammograms at the temperatures given in Table I. These experiments were carried out using method *2*. Adsorption at different temperatures and BSA concentrations was carried out in one cell. After transfer of the electrode for 30 s into the cell containing the buffer solution at the temperature at which the voltammogram was run the HMDE was transferred to the cell with buffer containing $5 \cdot 10^{-4}$M Co(III) or (II). The voltammograms at different temperatures were identical with those obtained under conditions given in Table I.

Quite generally, at each temperature the development of i_1 is considerably more rapid than that of i_2. The difference in rate of development is more pronounced the lower the temperature. As an example we report the following values obtained by method *1* at 4°C in a buffer which was $2\cdot5 \cdot 10^{-8}$M in BSA and $5 \cdot 10^{-4}$M in Co(III). Values of i_1 and i_2 in μA were 3·5 and 4·5 after 5 min, 10·5 and 18·5 after 10 min and 13 and 31 after 30 min with corresponding values of i_2/i_1 of 1·3, 1·8 and 2·5 respectively.

It is of importance to report that voltammograms were found to be identical whether run by method *1* or *2*. This means that the presence of Co(III) during the adsorption has no effect on the voltammogram.

TABLE I

Brdička Currents at HMDE at Various Temperatures after Covering the HMDE at 4, 21, 35 or 42°C with a Monolayer of BSA (method *1*)

Currents corrected for Co(III) or Co(II) currents.

Temperature of adsorption °C	i_1, μA	i_2, μA	i_2/i_1
4	12	30	2·5
21	14	~7	~0·5
35	14	~3	~0·2
42	13	~0	~0

Effect of concentration of Co(III): Using concentrations of $2{\cdot}5 \cdot 10^{-4}$, $5 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$M in Co(III) values of i_1 and i_2 were found somewhat less than proportional to concentration when a monolayer of BSA was adsorbed. In Table II we report only data obtained when the surface contained a monolayer of adsorbed BSA. At a given cobalt concentration i_1 remained virtually unchanged in the temperature range between 4°C and 35°C. At each temperature i_1 was found close to proportional to the square root of cobalt concentration. The same relation appears to hold for i_2 at 4°C, but at higher temperatures i_2 becomes increasingly less defined. Even though the values of i_2 in Table II at 21° and 35°C can be estimated only within $\pm 20\%$ it is evident that i_2 at a cobalt concentration of $1 \cdot 10^{-3}$M is very much greater at 21° and 35°C than at lower cobalt concentration.

Desorption studies were made using method *2* and an electrode which was partly as well as completely covered with a monolayer of BSA. After adsorption at different temperatures the HMDE was kept for varying short periods of time at $-1{\cdot}6$ V at the same temperature in the buffer without BSA and Co(III) before running a voltammogram at 21°C in a buffer $5 \cdot 10^{-4}$M in Co(III). The rate of desorption at $-1{\cdot}6$ V was found independent of the extent of coverage of the surface with BSA. It appeared that under all conditions the rate of decrease of i_2 at the various temperatures was considerably greater than that of i_1.

Use of Co(II) *instead of* Co(III): All experiments were repeated using Co(II) instead of Co(III). The various effects obtained with Co(II) were the same as those with Co(III).

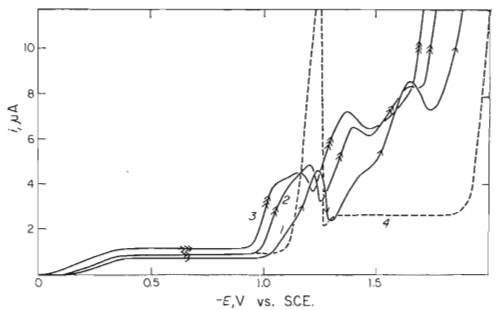

FIG. 1

Brdička Currents, i_1 and i_2, Run at Different Temperatures with Surface of HMDE Covered at 21°C with Monolayer of BSA

1 4°C; *2* 21°C; *3* 35°C; *4* buffer with $5 \cdot 10^{-4}$M-Co(III).

Voltammetry with modified samples of BSA: Samples of all products of modified BSA, composition of which is mentioned in the section Experimental were studied in a similar way as described for BSA. The behaviour of completely reduced BSA, $P(SH)_{35}$, was identical with that of native albumin except that values of i_1 and i_2 quite generally were 10% smaller than those obtained with native BSA. Qualitatively the same was found true in modified products in which part of the 17 —S—S— groups of BSA was eliminated or part of the —SH groups of $P(SH)_{35}$ was changed into inactive $—SCH_2COONa$ groups. With decrease of the number of —S—S— or —SH groups values of i_1 and i_2 decreased.

Polarography at the DME

In a previous paper[6], containing several references to the literature, polarograms of BSA at the DME under various conditions have been described and discussed. The effect of temperature had not been studied, but has been done in the present work. It should be realized that in ordinary polarography the extent of adsorption of protein on the electrode increases during the growth of a drop. Even when recording the currents when the drop falls (i_{max}) the extent of adsorption varies with the drop time, t, the mass of mercury per second, m, and of course, with the temperature. In the present study we have used native BSA and modified products of BSA. With native and modified BSA, i_2 was found to decrease when the temperature

TABLE II

Effect of Concentration of Co(III) at Various Temperatures on i_1 and i_2
Experimental conditions as Table I.

M-Co(III)	t, °C	i_1, μA	i_2, μA	i_2/i_1
$2{\cdot}5 \cdot 10^{-4}$	4	8·0	22	2·7
	21	9·0	~5·0	~0·5
	35	8·0	~1·0	—
$5 \cdot 10^{-4}$	4	14	30	2·1
	21	15	~8·5	~0·6
	35	14	~5	~0·3
$1 \cdot 10^{-3}$	4	21	41	2·0
	21	23	~23	~1
	35	22	~17	~0·8

was raised from 4°C to 21°C and to 35°C while i_1 increased with increasing temperature. As an example, we present in Fig. 2 polarograms obtained with a modified product of BSA, P[—$(SCH_2COONa)_7$, =$(S—S)_{14}$]. From the data presented in Table III, I, is seen that i_2/i_1 decreases with increasing temperature. Qualitatively the same results were found with various products of modified BSA, even though the values of i_1 and i_2 were found less than proportional to the sum of —S—S— and —SH groups.

TABLE III

Values of Polarographically Determined Currents i_1 and i_2 at 4, 21 and 35°C
For details see legend of Fig. 2.

t, °C	i_1, μA	i_2, μA	i_2/i_1
4	2·4	3·7	1·5
21	3·4	2·1	$0·6_2$
35	3·6	~1·3[a]	~0·3

[a] Very poorly defined.

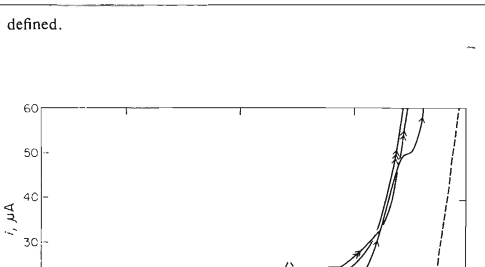

FIG. 2

Polarograms of $2·5 . 10^{-7}$M P[—$(SCH_2COONa)_7$ = $(S—S)_{14}$] in Buffer 0·1M in both Ammonia and Ammonium Chloride and $5 . 10^{-4}$M in Co(III).
Characteristics of DME: t = 4·5 s (at open circuit), m = 0·79 mg s^{-1}. Curves *1*—4 see Fig. 1.

In all experiments, both at the HMDE and DME, the Co(II) to Co(0) reduction waves shift with increasing temperatures to less negative potentials, while the potentials at which i_1 starts also become considerably less negative with increasing temperature (Fig. 1 and 2).

Presodium Currents, i_{ps}

In a previous paper[2], it has been shown that the plot of presodium currents (i_{ps}) at the HMDE *vs* potential exhibit a short plateau (Fig. 9 in ref.[2]). This plateau current has been shown to vary with the amount of adsorbed BSA on the electrode. In the present work, the HMDE was placed in the 0·1M ammonia buffer containing small concentrations of BSA. After various periods of adsorption at a given temperature the HMDE was placed in the protein-free buffer which was kept at different temperatures. The voltammograms were run at these temperatures.

The results can be summarized as follows; At the same extent of adsorption at 4°C as at 21°C i_{ps} run at 21°C was the same as when adsorbed at 21°C. On the other hand, with the same extent of adsorption the value of i_{ps} were found smaller when the voltammograms were run at 4°C than at 21°C. With increasing coverage the following values were obtained of i_{ps} (in μA) and of the ratio at 21°C to that at 4°C; 28/19 = 1·5, 45/30 = 1·5. 88/73 = 1·2. The last ratio corresponds to coverage of the surface with a monolayer. With increasing temperature the i_{ps} curves are shifted to slightly less negative potentials (Fig. 1 and 2).

Table IV

Effect of Temperature on the Voltammograms

The HMDE was kept a given time at 21°C in the buffer of pH 9·3 (at 21°C) which was 10^{-8}M in BSA. It was transferred to cell B with buffer, kept there for 30 s, and then transferred to cell C with buffer containing $5 . 10^{-4}$M Co(III) and of temperature as cell B. The HMDE was kept for the indicated time at —1·05 V (*vs* S.C.E.) and upon recycling for 2 s at E between +0·1 and —0·1 V and then scanned cathodically. Values of E at $(i_c)_{peak}$ varied from —1·45 to —1·50 V.

Time of adsorption in 10^{-8}M-BSA at 21°C min	Temperature in cells B and C, °C	Time at —1·05 V to give maximum i_c, min	i_c μA
1	4	1	37
2	4	1	54
1	21	2	165
2	35	2	230

CATALYTIC CURRENTS, i_c

The effect of a number of variables, but not of the temperature, upon the catalytic currents, i_c, observed when "active cobalt" had been deposited on the HMDE, have been described previously[4]. An exact interpretation of the effect of temperature on i_c cannot be given, because the amount of "active cobalt" formed and its stability vary with the temperature of its deposition in addition to the rate of the various reactions which account for the value of i_c. Since the extent of surface coverage of the HMDE also changes with the temperature of adsorption we report only some results obtained when the electrode contained the same amount of adsorbed BSA but at different temperatures of deposition of "active cobalt" and scanning. At each temperature of electrolysis the reactivity of the "active cobalt" deposited at $-1{\cdot}05$ V (*vs* S.C.E.) attained a maximum after 1 to 2 minutes of electrolysis and then decreased. As an illustration of the effect of temperature on the voltammograms we report in Table IV some results obtained (by method 2) when the HMDE contained the same amount of BSA adsorbed at 21°C. Comparable results were obtained when the adsorption was carried out at a temperature of 4°C as at 35°C.

DISCUSSION

Qualitatively the effects of temperature on i_{ps} and on i_c are as expected, both types of catalytic currents increase with increasing temperature. The interpretation of the effects of temperature on Brdička currents is more involved.

No irreversible denaturation occurs at 35°C, because at a given extent of adsorption at 35°C i_1 and i_2 are the same at 4°C as when the adsorption had occurred to the same extent at 4°C. The reverse is also true. Quite generally it can be stated that voltammograms vary with the temperature but at a given extent of adsorption and temperature are independent of the temperature of asorption between 4°C and 35°C. This behavior indicates that the area occupied on the mercury surface by the adsorbed protein is the same at temperatures between 4°C and 35°C but that the orientation of the sections at which the reactions occur which account for i_2 is such that with decreasing temperature more and more of the reactive groups are oriented in such a way that they can react with the electrons supplied by the electrode.

In agreement with Kuznetsov[7,8] we have concluded[2] that the rate of adsorption (actually of orientation) of the "i_2 section" is relatively slow as compared to that of the "i_1 section" and that the rate of its "desorption" (reorientation away from the electrode) at potentials close to $-1{\cdot}6$ V (*vs* S.C.E.) is relatively fast. Plots of i_2 *vs* temperature of running the voltammograms are smooth curves. These curves indicate that the rate of reorientation of the section of BSA reactive in the potential range of i_2 is rapid with change in temperature.

In a previous paper[2] a chain reaction has been proposed which accounts for the

large values of Brdička currents. The main reactions proposed were the reaction of the cobalt-protein complex to a cobalt(0) complex according to Eqs (*1*), the protonation of the cobalt(0) complex

$$P[—S_x^-Co(II)_y] + 2\,ye \rightarrow P[—S_x^-Co(0)_y] \qquad (1)$$

$$P[—S_x^-Co(0)_y] + x\,BH \rightarrow P[—(SH)_xCo(0)_y] + x\,B \qquad (2)$$

$$P[—(SH)_xCo(0)_y] + x\,e \rightarrow P[—S_x^-Co(0)_y] + \tfrac{1}{2}x\,H_2 \qquad (3)$$

(reaction *2*) and the reduction of the proton in sulfhydryl to hydrogen with reformation of Co(0) complex (reaction *3*). The termination reaction is the reduction of Co(II);

$$Co(II) + 2\,e \rightarrow Co(0) \xrightarrow{\text{with Hg}} Co(0)\ \text{amalgam}\,. \qquad (4)$$

It is of major importance to mention that the same Brdička currents are obtained by method *1* as well as by method *2*. In method *1* the Co(III) or Co(II) complex with the protein is adsorbed on mercury from the solution. In method *2* the HMDE with adsorbed protein is placed in the Co(III) or (II) containing buffer and the voltammograms are the same as those by method *1*. Quite generally, only the sulfhydryl group has been considered as the ligand forming the complex. However, it is quite evident that other ligands in BSA or modified BSA participate in the complex formation because —SH—free, but —S—S— containing protein yield the same Brdička currents as the protein containing the equivalent number of —SH groups.

It is also evident that cobalt complexes with mercaptide ions are formed in the potential range at which Brdička currents are observed because in the absence of Co(III) or (II) Stankovich and Bard[9] found that at the HMDE only about one disulfide group of BSA is rapidly reduced to sulfhydryl.

In order to account for the large decrease of i_2 (Brdička current) with increasing temperature it must be concluded that the section of the protein molecule which yields the large value of i_2 at 4°C is oriented at the electrode in such a way that the cobalt(II) mercaptide section can react with electrons supplied by the electrode, but that this section is continuously reoriented away from the surface with increasing temperature.

The polarographic curves (Fig. 2) at the DME are interpreted only in a qualitative way as the amount of BSA adsorbed during the/formation of a drop increases with increasing temperature.

In order to throw further light on the various reactions which play a major role on the mechanism of reactions which occur at potentials at which Brdička currents and i_c occur we had plans to study these currents with lower molecular weight

sulfhydryl and/or disulfide containing compounds, including peptides. Unfortunately, because of discontinuation of the grant the present wotk has been discontinued.

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